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ORIGINAL ARTICLE

HLA Antigens Shed from the Surface of Synthetic or Naturally Occurred Platelet-Derived Microparticles During Storage of Platelet Concentrate

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Abstract The demand for standard platelet concentrates (PCs) has continued to increase in the recent years. Infusible platelet membranes (IPM) prepared from new or outdated human platelets have been developed as an alternative to standard PCs, with the additional advantage of long shelf life and increased viral safety. Reduction of HLA antigens on the IPM has been assigned as one of the probable advantages of this product. In re-examining this issue, we studied the existence of HLA class I on the surface of IPM microparticles. In comparison we also surveyed HLA expression on the surface of the naturally occurred platelet-derived microparticles (nPMPs) during 7 days storage. Intended for producing IPM, PCs obtained from Iranian blood transfusion organization were lysed; virally inactivated with wet heat in the presence of a heat stabilizer and then sonicated. IPMs were separated using centrifugation and liquid-stored in 4°C. The expression of HLA class I antigens was surveyed using flow cytometry technique. HLA molecules were present on the microparticles. Shedding of HLA antigens was demonstrated from the surface of the both liquid-stored IPM and nPMPs during storage. Storage of IPM in 4°C was accompanied with significant reduction of HLA molecules. It seemed that achievement of HLA-free IPM could be impossible unless chloroquine treated platelets were used to prepare these microvesicles.

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Introduction

Platelets have been available for routine clinical use for over four decades. The most widely used preparation is a concentrate stored in liquid state at 22°C. Such liquid-stored platelets have a shelf-life of 3–5 days; a time governed largely by the increased risk of bacterial septic, and other reactions, associated with longer storage [1, 2]. Despite the improvements in PC preparations, adverse effects in recipients continue to be reported [3]. Much effort, therefore, has been expended on the development of novel platelet products, substitutes and alternatives for the treatment of the bleeding thrombocytopenic patients [4–6].

Many new approaches have been explored experimentally to produce hemostatically active platelet products that are capable of long term storage. These include: platelet storage in the frozen state; storage in the liquid state in the cold (4°C); rehydrated lyophilized platelets; platelet membrane microparticles; and photochemical treatment capable of pathogen inactivation [7–9]. In addition to platelet-derived products, investigators have explored various approaches to create artificial substitutes that are capable of in vivo hemostasis [10].

Microparticles (MP) are small plasma membrane vesicles shed from cells upon their activation or apoptosis. Platelet-derived microparticles (nPMPs) constitute the majority of the pool of MPs circulating in the blood [11]. nPMPs can express and transfer functional receptors from platelet membranes. The influence of nPMPs on the angiogenic activity of endothelial progenitor cells was recently shown [12].

Infusible platelet membranes (IPM) prepared from new or outdated human platelets have been developed as an alternative to standard PCs. These microparticles have additional advantages like long shelf life, increased viral safety and decreased adverse reactions due to the removal of plasma [13–16].

Platelets express HLA class I molecules to a less extent than peripheral mononuclear cells. Taking into account the number of platelets, it can be estimated that platelets carry approximately two thirds of the HLA molecules from blood. The origin of platelet-surface class I molecules has been discussed intensively. Different groups have shown that HLA-A and -B antigens of platelets are integral membrane constituents. Other experiments, based on chloroquine treatment or co-incubation of platelets with allogenic plasma, suggest that most HLA molecules are adsorbed from the plasma. Altogether, these data argue for co-expression of integral membrane and adsorbed HLA molecules on platelets, although the contribution of these 2 types is controversial [17]. Studies related to the production of IPM and surveys of its features are very limited. Some of them have been reported that HLA class I molecules can't be detected on the IPM using a monoclonal antibody but a polyclonal antibody specific to denatured HLA class I antigens can distinguish HLA in the IPM preparations [13]. HLA class I is the main eliciting molecule in refractoriness to platelets thus removal of HLA from platelets or IPM could be extremely valuable. In re-examining these issues, we studied the IPM production and the retention of HLA class I on these microvesicles.

Materials and Methods

Preparation of IPM and Isolation of nPMPs

Thirty-five units of platelet concentrates (2–4 days old) prepared by the Iranian Blood Transfusion Organization were used to produce IPM after informed consent for the following studies was obtained from donors. Platelets were used to produce IPM as it was reported before [18]; briefly; these cells were lysed by replicate freezing and thawing for 3 times, virally inactivated by moist heat at 60°C for 20 h with different concentrations of sodium octanoate (0, 0.1, 0.25, 0.4, 0.6 M). IPM were divided into vials and kept in refrigerator. Beside, for preparing nPMPs from platelet concentrates, platelets from 5 units of platelet concentrates were transferred into 15 ml falcon tubes. The tubes were centrifuged at $150\times g$ for 10 min to remove remaining white and red blood cells. The supernatant were separated and then depleted from platelets using centrifugation at $1{,}200\times g$ for 10 min. Finally isolation of nPMPs from plasma was carried out using centrifugation at $15{,}000\times g$ for 15 min.

HLA Class I Expression

We determined HLA class I antigen expression on the prepared IPM and also the platelet-derived microparticles during storage. For this purpose, 6 µl of FITC-conjugated anti-HLA class I antibodies (W6/32, Abcam, UK) were added to different tubes each included 100 µl of a suspension of nPMPs or the prepared IPM (500 µg/ml) that prepared in different concentrations of the stabilizer. The tubes were left for 40 min at RT and then studied by flow cytometry. To be sure of the specificity of the results, nonspecific antibody background binding was determined with the appropriately labeled isotypic control immunoglobulin IgG. Flow cytometric analysis was carried out by the flow cytometer (Partec-pasIII, Germany).

Statistical Analysis

Statistical differences between the percent of HLA expression in IPM microparticles were tested by Mann–Whitney test. A P-value of less than 0.05 was considered significant.

Results

HLA Class I Retention on the IPM Microparticles

HLA class I antigens were present on the surface of IPM. As mentioned under Materials and Methods, different concentrations of the heat stabilizer were used in the heat treatment stage of inactivating viral contaminants. The results showed that there was no significant differences between the extent of detected HLA (P-value >0.05) in the presence or absence of sodium octanoate. The results of flow cytometry technique demonstrated the reduction of HLA antigens during 4 months storage (Fig. 1; Table 1) and the differences between the results for fresh, 1-month and 4-month old IPM were distinguished significant (P-value <0.05). These results implied that the HLA class I antigens were shed from the surface of IPM during the storage period.

HLA Class I Retention or Shedding from nPMPs

In the platelet concentrate, the naturally occurred nPMPs showed the mean HLA content of 70.01 ± 22.5, 56.34 ± 21.1 and $36.56 \pm 24.8\%$ at the days 2, 4 and 7 of storage. So HLA level had a reductive pattern during the storage time on the nPMPs. The differences were significant between the expression level at the days 2 and 4 or the days 2 and 7 (P-value $= 0.03$) of storage.

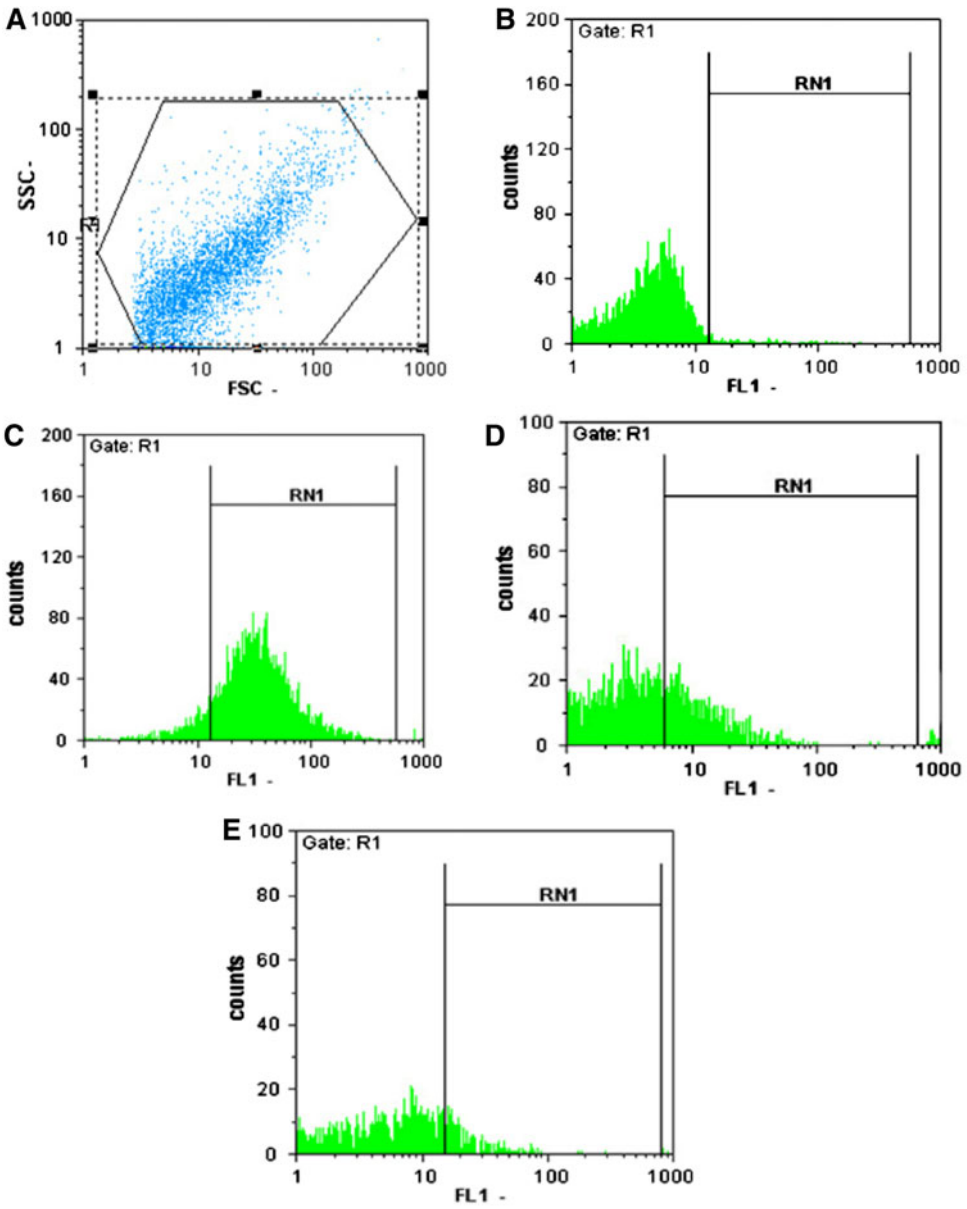

Fig. 1 Flow cytometry plot. IPM was prepared in 0.25 M concentration of sodium octanoate and liquid-stored in 4°C for 4 months. Expression of HLA class I was analyzed during this time. **a** The microparticles were gated, **b** the isotypic control immunoglobulin IgG, **c** represented the HLA levels in the freshly prepared IPM, **d** represented the HLA levels in the surface of IPM 1 month after preparation, **e** represented the HLA levels in the surface of IPM 4 months after preparation

Table 1 The extent of HLA class I expression in liquid-stored IPM prepared in several concentrations of sodium octanoate during storage for 4 months

Extent of HLA expression (%) for	Concentration of sodium octanoate			
	0 M	0.1 M	0.25 M	0.4 M
Freshly prepared IPM	73.2	71	70	63.4
IPM, 1 month after preparation	23.1	20	24.1	19.1
IPM, 4 month after preparation	3.8	7.5	8.2	9.7

Discussions

The exact function of platelets' HLA class I molecules is still unknown. However, it has been proposed that they are implicated in alloimmunization following multiple platelet transfusions. Production of anti-HLA class I alloantibodies has been reported in about half the patients receiving non-leukocyte-depleted thrombocyte concentrates, and refractoriness to platelet transfusion occurs secondarily [3].

Platelet microparticles are platelet membrane microvesicles that were first described over 30 years ago. Such platelet microparticles form spontaneously during the storage of platelets and have been found in platelet concentrates, fresh-frozen plasma, and cryoprecipitate. These microparticles, like intact platelets, possess procoagulant activity; adhere to the vascular subendothelium; and enhance platelet adhesion [19, 20]. Taking into account the functionality of platelet microparticles and their hemostatic properties similar to those of intact platelets, it was reasonable for investigators to examine the potential of microparticles as a possible platelet substitute.

Platelet microparticles can be induced synthetically. A microparticulate human platelet preparation has been developed by Cypress Bioscience, SanDiego, California. This company's infusible platelet membranes (IPM) were prepared from outdated platelet concentrates. This IPM, which consisted of spherical vesicles with a diameter of approximately 0.6 μm, has been shown to contain various procoagulant phospholipids [19].

In this study we surveyed the IPM production and its surface retention of HLA class I molecules and also the effect of the heat stabilizer and storage duration on their maintenance. The results showed the existence of HLA class I on the IPM. The extent of HLA antigens was reduced during the storage of liquid-stored form of IPM. There were differences in this regard with the previous study of Chao et al. [13] who reported that HLA class I was not detectable on the IPM by a monoclonal antibody after the heating step (60°C for 20 h) but they had another observation in which a polyclonal antibody against denatured HLA class I antigen reacted with IPM before and after heat treatment. Their observation showed that denaturation was occurred in HLA molecules following heating.

We followed and detected the HLA antigens using a monoclonal antibody specific to the native HLA class I antigens after heat treatment. The results demonstrated that application of a heat stabilizer (sodium octanoate) had no detectable effect on the levels of HLA molecules. Whereas we found that the maximum binding of IPM to vWF occurred with the IPM prepared in the 0.25 or 0.4 M concentration of sodium octanoate. So, the ability of IPM to bind to vWF was dependent on the concentration of the stabilizer [18].

HLA antigens were shed from the surface of the IPM with time and the main reduction of HLA took place after 4 months storage of liquid-stored IPM. HLA molecules also were shed from the nPMPs during 7 days storage of platelet concentrate.

The production of IPM with low levels of HLA class I could be feasible. Considering the methods that could reduce HLA molecules from the surface of platelets like treatment with chloroquine [21], it sounds that generation of IPM from these treated PCs could be accompany with lower levels of HLA.

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Conflict of interest None of the authors have any conflicts of interest to declare.

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